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Application of:

Leong Ng

Serial No: 10/719,695

Filed: November 21, 2003

For: Bodily Fluid Markers of Tissue
Hypoxia

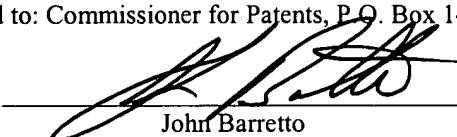
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Art Unit: 1645

Attorney Docket No. ISA-012.01

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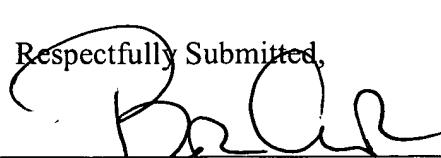
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Sir:

Attached hereto are certified copies of GB 0227179.9 filed November 21, 2002, and GB 0322390.6 filed September 24, 2003, priority documents for the above-referenced application. Should there be any questions after reviewing this submission, the Examiner is invited to contact the undersigned at 617-832-1294.

Respectfully Submitted,


Beth E. Arnold
Reg. No. 35,430

Date: September 8, 2004

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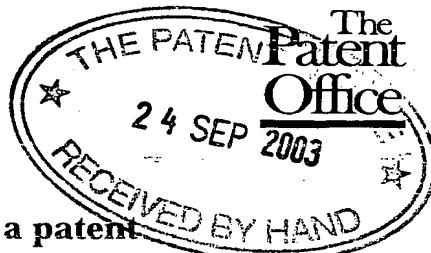
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1/77
25SEP03 E839696-1 D00056
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The University of Leicester
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LE1 7RH

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

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800379 0001

4. Title of the invention

Bodily fluid markers of tissue hypoxia

5. Name of your agent (if you have one)

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Description 26

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Nicholas Lee
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Bodily fluid markers of tissue hypoxia

Field of the Invention

5 This invention relates to the detection and measurement in bodily fluids of one or more markers of tissue hypoxia or of a clinical syndrome attributable to tissue hypoxia in a mammalian subject. In particular, this invention relates to the detection of Oxygen Regulated Protein (ORP150) or peptide fragments derived therefrom, alone or in combination with additional markers, in the diagnosis and/or prognosis of
10 tissue hypoxia or of a clinical syndrome attributable to tissue hypoxia in a mammalian subject.

Background to the invention

15 Ischaemic heart disease and heart failure are major health problems in the world but the means effectively to diagnose and manage these conditions at the moment are limited.

Chronic heart failure

20 Chronic heart failure (CHF) is a common clinical syndrome which is an increasingly important health care issue in industrialised societies with elderly populations. Hospitalisation rates for heart failure have increased markedly over the last 20 years and CHF is associated with poor prognosis and quality of life. The direct costs of CHF account for approximately 1-2% of health care expenditure, the vast majority
25 being related to hospital admissions.

Chronic heart failure is most often the result of left ventricular systolic dysfunction (LVSD). Screening studies from Glasgow (McDonagh, *et al*, *Lancet* 1997; **350**: 829-8331) and Birmingham (Davies, *et al*, *Lancet* 2001; **358**: 439-444) indicated
30 prevalence of rates of definite LVSD of 2.9% and 1.8% respectively. In both studies, the condition was asymptomatic in half of the cases. The identification of patients with LVSD allows the prescription of appropriate therapy which for the individual

patient improves quality of life and prognosis. Echocardiography is currently the most frequently used investigation for the diagnosis of LVSD and heart failure.

The pathophysiology of CHF involves activation of many neurohormonal systems, 5 including the catecholamine, renin-angiotensin, endothelin, atrial and brain natriuretic peptide systems. Some of these systems are activated in an adaptive fashion (the natriuretic peptide systems); others although adaptive under acute conditions become maladaptive especially when maintained in the chronic state (endothelin, renin-angiotensin and catecholamine systems). An increased secretion of the natriuretic 10 peptide hormones has been exploited as a means for diagnosis of CHF (McDonagh *et al*, *Lancet* 1998; 351:9-13; Hobbs, *et al*, *Br Med J* 2002; 324: 1498-1502). For detection of LVSD, brain natriuretic peptide (BNP) is a better diagnostic tool than N-terminal pro-atrial natriuretic peptide (N-ANP) (McDonagh *et al*, *Lancet* 1998; 351:9-13). In addition, another peptide derived from the precursor of BNP, namely N- 15 terminal proBNP (N-BNP) is also a reasonable alternative for the identification of LVSD (Hobbs, *et al*, *Br Med J* 2002; 324: 1498-1502). In both cases, the negative predictive values of the tests are high, suggestive of their utility in the exclusion of CHF. In many cases of CHF, the aetiology is ischaemic heart disease. In addition, a 20 reduced cardiac output over a chronic period would lead to tissue hypoperfusion and a relative tissue hypoxia.

However, an indicator in the plasma that is induced and secreted when tissues are hypoxic would have great utility in the diagnosis and prognosis of heart disease, and have further utility in the monitoring of such diseases.

25

Ischaemic heart disease

Ischaemic heart disease is a major health burden in developed countries, and its main aetiology is atherosclerosis. Accumulation of lipid, especially oxidised or modified LDL, together with macrophages and other cells leads to plaque growth and 30 instability.

Oxygen Regulated Protein ORP150 and its role as a molecular chaperone in the Endoplasmic Reticulum

Oxygen regulated protein (ORP150) is a chaperone endoplasmic reticulum associated protein that was originally cloned from astrocytes subjected to hypoxia (Kuwabara, *et al, J Biol Chem* 1996; **271**: 5025-32). The induction of this protein in rat astrocytes, human aortic vascular myocytes and mononuclear leucocytes showed specificity for hypoxia but not other stressful stimuli such as glucose deprivation, hydrogen peroxide, tunicamycin or heat shock (Kuwabara, *et al, J Biol Chem* 1996; **271**: 5025-32; Tsukamoto, *et al, J Clin Invest* 1996; **98**: 1930-41). In addition, tissue extracts prepared from human atherosclerotic lesions demonstrated increased expression of ORP150 mRNA and protein, with most of the mRNA found in macrophages (Tsukamoto, *et al, J Clin Invest* 1996; **98**: 1930-41). ORP150 may function as a molecular chaperone in cells of the atherosclerotic vessel wall, present mainly in the endoplasmic reticulum (ER) of cells subjected to hypoxia (Tsukamoto, *et al, J Clin Invest* 1996; **98**: 1930-41). Its expression may be further enhanced by the presence of oxidised or acetylated light density lipoproteins (LDL), a known risk factor for vascular disease (Tsukamoto, *et al, J Clin Invest* 1996; **98**: 1930-41). Its functions include a protective effect against hypoxia induced damage, since reducing its expression with antisense oligonucleotides leads to enhanced susceptibility of mononuclear phagocytes to hypoxic damage (Tsukamoto, *et al, J Clin Invest* 1996; **98**: 1930-41).

The cloning of human ORP150 recently demonstrated a deduced amino acid sequence of 999 residues, with an endoplasmic reticulum (ER) retention signal at the C terminus (Ikeda, *et al, Biochem Biophys Res Commun* 1997; **230**: 94-9). It has some homology to other stress induced proteins such as glucose regulated protein (GRP170) and heat shock protein (HSP70). Its location in the ER suggests a role as a molecular chaperone for protein folding and maturation, especially in hypoxic conditions. For example, kidney cells ORP150 antisense transformants showed delayed maturation of glycoprotein GP80, the latter accumulating in the ER, thus indicating a role for ORP150 in protein maturation and transport within ER of cells

under conditions of energy deprivation (Bando, *et al*, *Am J Physiol Cell Physiol* 2000; **278**: C1172-82).

Evidence for a function of ORP150 in vascular disease comes from observations on a 5 stroke model in mice (Matsushita, *et al*, *Brain Res Mol Brain Res* 1998; **60**: 98-106; Tamatani, *et al*, *Nat Med* 2001; **7**: 317-23). In a mouse model of cerebral ischaemia, there was rapid induction of ORP150 mRNA and protein in the hypoxic neurons, even within the ischaemic and energy depleted zones (Matsushita, *et al*, *Brain Res Mol Brain Res* 1998; **60**: 98-106). In ischaemic human brains, although ORP150 10 expression in neurons was only sparingly induced, there was a significant induction of ORP150 in astrocytes (Tamatani, *et al*, *Nat Med* 2001; **7**: 317-23). Neurones overexpressing ORP150 were resistant to hypoxic stress and mice genetically engineered to overexpress ORP150 in their neurons had smaller strokes under ischaemic stress (Tamatani, *et al*, *Nat Med* 2001; **7**: 317-23). Cytoprotection was 15 associated with suppressed caspase-3-like activity and enhanced brain-derived neurotrophic factor (BDNF), indicating a role for ORP150 in cytoprotection under hypoxic conditions (Tamatani, *et al*, *Nat Med* 2001; **7**: 317-2310). In addition, astrocytes or cell lines transfected to overexpress ORP150 antisense RNA were more susceptible to hypoxic stress, leading to apoptosis (Tamatani, *et al*, *Nat Med* 2001; **7**: 20 317-23; Ozawa, *et al*, *J Biol Chem* 1999; **274**: 6397-404).

Other evidence supporting a role for ORP150 in vascular disease lies in its association with angiogenesis (Tsukamoto *et al*, *Lab Invest* 1998; **78**: 699-706; Ozawa *et al*, *Cancer Res* 2001; **61**: 4206-13; Ozawa *et al*, *J Clin Invest* 2001; **108**: 41-50). 25 ORP150 is expressed in tumours, with high expression in the cells invading host tissue (Tamatani, *et al*, *Nat Med* 2001; **7**: 317-23). Invasion is often associated with the co-expression of vascular endothelial growth factor (VEGF, a recognised angiogenic factor) and ORP150 (Ozawa *et al*, *Cancer Res* 2001; **61**: 4206-13), and tumour cells transfected with antisense ORP150 are less invasive (Ozawa *et al*, *Cancer Res* 2001; **61**: 4206-13), with defective maturation of VEGF leading to its 30 accumulation in the ER (Ozawa *et al*, *Cancer Res* 2001; **61**: 4206-13). In wounds, neovascularisation is associated with expression of both VEGF and ORP150 (Ozawa

et al, J Clin Invest 2001; 108: 41-50). Local administration of adenovirus bearing ORP150 leads to enhanced wound repair, new vessel formation and VEGF expression at the site (*Ozawa et al, J Clin Invest 2001; 108: 41-50).* Macrophages engineered to reduce ORP150 expression have defective VEGF maturation, the latter accumulating in the ER, whereas overexpression of ORP150 leads to successful export and secretion of the VEGF product (*Ozawa et al, J Clin Invest 2001; 108: 41-50).* Enhanced angiogenesis with ORP150 expression would further support its role a cytoprotective agent from its other intracellular effects on apoptosis suppression under hypoxic stress.

10

Summary of the invention

In a first aspect, the present invention provides a method for screening, diagnosis or prognosis of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for determining the stage or severity of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for identifying a mammalian subject at risk of developing tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, or for monitoring the effect of therapy administered to a mammalian subject having tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, said method comprising:

measuring the level of a first marker in a sample of bodily fluid from said mammalian subject, wherein said first marker is oxygen regulated protein (ORP150) or a fragment thereof.

In conditions of tissue hypoxia, there may be increased expression of ORP150 in tissues. The ORP150 protein is associated with endoplasmic reticulum and is not expected to be secreted. However, the present inventors have unexpectedly shown that ORP150 or peptide fragments derived from ORP150 can be detected extracellularly (in human plasma using an immunoassay) and have been able to show its utility as a marker for the diagnosis and prognosis of CHF. Furthermore ORP150 or peptide fragments thereof have been detected in plasma at higher levels in patients who have heart failure and ischaemic heart disease (e.g. myocardial infarction) than

normal subjects. Measurement of these markers is therefore useful as a diagnostic aid for presence of heart failure and for assessing the severity of heart failure.

5 ORP150 or peptide fragments thereof may also be useful in assessing the prognosis of patients with ischaemic heart disease or acute coronary syndromes; especially after myocardial infarction, levels of the peptide are elevated in patients at risk of increased mortality or readmission with heart failure.

10 As used herein, the term "clinical syndrome indicative of tissue hypoxia" refers to a disease state or condition at any stage of progression, which occurs as a consequence of tissue hypoxia or which results in tissue hypoxia. Thus, it includes conditions such as ischaemia; the process of ischaemia; tissue injury leading to cell necrosis; chronic heart failure; acute occlusion of the coronary circulation, such as in ischaemic heart disease, myocardial infarction and other acute coronary syndromes (e.g. non ST 15 elevation myocardial infarction and unstable angina); clinical syndromes attributable to atherosclerosis; stroke; aortic aneurysm; peripheral vascular disease; chronic lung disease; and tumour.

20 In the present invention, a fragment of ORP150 is a fragment of the ORP150 protein which has an amino acid sequence which is unique to ORP150. The amino acid sequence for human ORP150 is provided in Figure 16 (NCBI database Accession AAC50947, Accession NP_006380). The fragment may be as few as 6 amino acids, although it may be 7, 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids. In one embodiment, the fragment comprises or consists of the sequence 25 LAVMSVDLGSESM. The fragment may have a molecular weight in the range of from 6 to 8, 6.5 to 7.5, 6.7 to 7.4, 1 to 4, 1.5 to 3.5, or 1.8 to 3.3 kD. The molecular weight may be determined by means known to those skilled in the art such as gel electrophoresis or size exclusion chromatography.

30 In the present invention, ORP150 can be detected in plasma or other bodily fluids such as interstitial fluid, urine, whole blood, saliva or serum. The mammalian subject may be a human.

The level of ORP150 or fragment thereof may be compared with the level of ORP150 or fragment thereof from one or more mammalian subjects free from tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, or with a previously determined reference range for ORP150 or fragment thereof in such mammalian subjects. In this way, the levels can be compared with reference levels determined from population studies of subjects free from the condition in question to provide a diagnosis or prognosis. Such subjects may be matched for age and/or gender. Where the present invention is concerned with monitoring the effect of therapy administered to a mammalian subject having tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, the measured level of ORP150 or fragment thereof can be compared with a base level for the subject. The base level may be determined prior to commencement of the therapy. Deviations from this base level indicate whether there is an increase or decrease of hypoxia and hence whether the therapy is effective. An increased level of ORP150 or fragment thereof indicates tissue hypoxia and *vice versa*.

In the method of the present invention, a second marker indicative of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject may be measured. The clinical syndrome indicative of tissue hypoxia may be heart failure or ischaemic heart disease. In this instance, the method of the present invention may further comprise measuring the level of a second marker indicative of heart failure or ischaemic heart disease. The second marker may be a natriuretic peptide, such as brain natriuretic peptide (BNP) or N-terminal pro-brain natriuretic peptide (N-BNP). The release of proBNP (the intact precursor to the two circulating forms, BNP (the active peptide) and N-BNP (the inactive peptide)) from cardiac myocytes in the left ventricle and increased production of BNP is triggered by myocardial stretch, myocardial tension, and myocardial injury. ORP150 or peptide fragments thereof may be useful in combination with the natriuretic peptides (e.g. N-terminal proBrain natriuretic peptide or N-BNP) in assessing the prognosis of patients with ischaemic heart disease or acute coronary syndromes; after myocardial infarction, the combination of peptides is useful in risk stratification of patients with respect to mortality.

In a manner similar to the first marker, ORP150 or a fragment thereof, the level of the second marker may be compared with the level of the second marker from one or more mammalian subjects free from tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, or with a previously determined reference range for the second 5 marker in mammalian subjects free from tissue hypoxia or a clinical syndrome indicative of tissue hypoxia.

The respective levels of the first and second markers may be measured using an immunoassay. Such assays may be competitive or non-competitive immunoassays. 10 Such assays, both homogeneous and heterogeneous, are well-known in the art, wherein the analyte to be detected is caused to bind with a specific binding partner such as an antibody which has been labelled with a detectable species such as a latex or gold particle, a fluorescent moiety, an enzyme, an electrochemically active species, etc. Alternatively, the analyte may be labelled with any of the above detectable 15 species and competed with limiting amounts of specific antibody. The presence or amount of analyte present is then determined by detection of the presence or concentration of the label. Such assays may be carried out in the conventional way using a laboratory analyser or with point of care or home testing device, such as the lateral flow immunoassay as described in EP291194.

20 In one embodiment, an immunoassay is performed by contacting a sample from a subject to be tested with an appropriate antibody under conditions such that immunospecific binding can occur if the marker is present, and detecting or measuring the amount of any immunospecific binding by the antibody. Any suitable 25 immunoassay can be used, including, without limitation, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, 30 complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays.

For example, a marker can be detected in a fluid sample by means of a two-step sandwich assay. In the first step, a capture reagent (e.g., an anti-marker antibody) is used to capture the marker. The capture reagent can optionally be immobilised on a 5 solid phase. In the second step, a directly or indirectly labelled detection reagent is used to detect the captured marker. In one embodiment, the detection reagent is an antibody. In another embodiment, the detection reagent is a lectin.

The term "antibody" as used herein refers to immunoglobulin molecules and 10 immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that specifically binds an antigen. The immunoglobulin molecules useful in the invention can be of any class (e.g., IgG, IgE, IgM, IgD and IgA) or subclass of immunoglobulin molecule. Antibodies includes, but are not limited to, polyclonal, monoclonal, bispecific, humanised and chimeric 15 antibodies, single chain antibodies, Fab fragments and F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

One antibody useful for detecting ORP150 may recognise the sequence 20 LAVMSVDLGSESM. Other suitable antibodies are available commercially from Immuno-Biological Laboratories Co. Ltd, 1091-1 Naka, Fujioka-shi, Gunma, 375-0005, Japan.

The present invention also provides a kit for carrying out the methods of the 25 invention.

Also provided is a kit for screening, diagnosis or prognosis of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for determining the stage or severity of tissue hypoxia or of a clinical syndrome 30 indicative of tissue hypoxia in a mammalian subject, for identifying a mammalian subject at risk of developing tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, or for monitoring the effect of therapy administered to a mammalian subject

having tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, said kit comprising:

instructions for taking a sample of bodily fluid from said mammalian subject; and

5 one or more reagents for measuring the level of oxygen regulated protein (ORP150) or fragment thereof in the sample.

The one or more reagents may comprise an antibody that is immunospecific for the first marker, as is described above. The kit may further comprise one or more

10 reagents for measuring the level of a second marker indicative of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, especially when the clinical syndrome indicative of tissue hypoxia is heart failure or ischaemic heart disease, as described above.

15 In addition, a kit of the invention may optionally comprise one or more of the following: (1) instructions for using the kit for screening, diagnosis or prognosis of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for determining the stage or severity of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for identifying a

20 mammalian subject at risk of developing tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia, or for monitoring the effect of therapy administered to a mammalian subject having tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia; (2) a labelled binding partner to any antibody present in the kit; (3) a solid phase (such as a reagent strip) upon which any such antibody is immobilised; 25 and (4) a label or insert indicating regulatory approval for diagnostic, prognostic or therapeutic use or any combination thereof. If no labelled binding partner to the or each antibody is provided, the or each antibody itself can be labelled with a detectable marker, e.g., a chemiluminescent, enzymatic, fluorescent, or radioactive moiety.

30 ORP150 is hyperexpressed in the vessel wall of atheromatous plaques (Tsukamoto, *et al, J Clin Invest* 1996; **98**: 1930-41), present mainly in the macrophages. Since the inventors have demonstrated increased ORP150 in plasma from patients with heart

failure and ischaemic heart disease (of which the main cause is atherosclerosis), it is likely that patients with other clinical syndromes attributable to atherosclerosis such as ischaemic stroke, aortic aneurysm, peripheral vascular disease or other acute coronary syndromes will have increased ORP150 in their plasma, and the marker can 5 be used as an indicator of the presence or severity of these conditions. Similarly, patients with chronic lung disease where tissues may be hypoxic would also be expected to have increased ORP150 in their plasma. Currently, there is no easy way of measuring pO₂ in patients with lung disease, who for example are on home oxygen therapy, and a further use of ORP150 measurements is in the monitoring of oxygen 10 therapy in such patients. As invasive tumours may overexpress ORP150 (Tsukamoto *et al*, *Lab Invest* 1998; **78**: 699-706; Ozawa *et al*, *Cancer Res* 2001; **61**: 4206-13; Asahi *et al*, *BJU Int* 2002; **90**: 462-6) as a chaperone for vascular endothelial growth factor, it can be used as a tumour marker, especially of invasive tumours that may be relatively hypoxic in their central regions. Such use would extend to diagnosis and 15 also for the monitoring of treatment of these invasive tumours.

In a further aspect, the invention provides the use of oxygen regulated protein ORP150 or peptide fragments thereof as a marker of tissue hypoxia or as a marker of a clinical syndrome attributable to tissue hypoxia in a mammalian subject. The 20 clinical syndrome attributable to tissue hypoxia may be due to heart failure, ischaemic heart disease, atherosclerosis, ischaemic stroke, aortic aneurysm, peripheral vascular disease, lung disease or tumour growth.

In another aspect, the invention provides the use of ORP150 or peptide fragments 25 thereof as a cardiac marker for use as a diagnostic tool in the determination of the presence or severity of heart failure or ischaemic heart disease in a mammalian subject. ORP150 or peptide fragments thereof may be used in combination with a further marker indicative of heart failure or ischaemic heart disease. The further marker may be a natriuretic peptide, such as brain natriuretic peptide (BNP) or N-terminal probrain natriuretic peptide (N-BNP). 30

The invention also provides a method for the determination of the presence or severity of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia wherein a sample of bodily fluid from a mammalian subject is measured for the presence of ORP150 or peptide fragments thereof. The clinical syndrome indicative of tissue hypoxia may be heart failure or ischaemic heart disease, and the bodily fluid may be plasma. A diagnosis or prognosis may be made based upon the result obtained compared to that obtained from a healthy individual or individuals. The measurement may be carried out by use of an immunoassay.

5

10 The invention also provides a method of detection of oxygen regulated protein (ORP150) or a fragment thereof, said method comprising obtaining a sample of bodily fluid from a mammalian subject and performing a measurement on said sample to indicate the presence and/or amount of said (ORP150) or a fragment thereof.

15 Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

EXAMPLES

20 The invention will now be described in more detail in the following non-limiting examples. Reference is made to the accompanying drawings in which:

Figure 1 shows a standard curve for the ORP150 peptide competitive immunoassay.

25 A patient's plasma extract (solid circles joined by solid line) was diluted in two fold steps, showing parallelism with the standard curve. Two patients' urine extracts were also diluted in two fold steps (hollow triangles joined by dotted lines), again demonstrating parallelism with the standard curve.

30 Figure 2 shows the results of size exclusion chromatography with analysis of the fractions for ORP150. The points of elution of markers for 150 kd, 20 kD and 6.5 kD

are indicated by arrows. Three peaks of immunoreactivity for ORP150 are evident at 150, approximately 7 and approximately 3 kD.

5 Figures 3a and 3b are box plots of log transformed plasma N-BNP and ORP150 levels respectively in normal subjects, heart failure patients and patients with myocardial infarction.

10 Figures 4a and 4b are box plots of log transformed plasma N-BNP and ORP150 levels respectively in normal subjects and heart failure patients of both gender.

15 Figures 5a and 5b show the relationship of plasma N-BNP and ORP150 respectively with severity of heart failure (as judged by the NYHA class) in males and females.

20 Figure 6 shows a Receiver Operating Characteristic curve for diagnosis of heart failure, using N-BNP or ORP150 alone, and using the prognostic index derived from a logistic model with a combination of N-BNP and ORP150.

25 Figure 7 shows the relationship of plasma N-BNP and ORP150 to Killip class in patients after myocardial infarction.

30 Figure 8 shows the relationship of plasma N-BNP and ORP150 to left ventricular function as assessed by echocardiography in patients after myocardial infarction. Ventricular dysfunction is classified as normal, mild, moderate or severe impairment.

Figure 9 shows a comparison of the levels of N-BNP and ORP150 to the clinical outcome of death in patients after myocardial infarction.

Figure 10 shows a comparison of the levels of N-BNP and ORP150 to the clinical outcome of rehospitalisation with heart failure in patients after myocardial infarction.

Figures 11a and 11b show a survival analysis of patients following myocardial infarction, stratifying patients as below or above the median value of plasma N-BNP or of ORP150 respectively.

5 Figure 12 shows a survival analysis of patients following myocardial infarction, stratifying patients as having both plasma levels of N-BNP and ORP150 below or above the median, and an intermediate group in which either peptide is above their respective medians.

10 Figure 13 shows a comparison of the levels of N-BNP and ORP150 to the clinical outcome of death in patients after unstable angina/Non-ST elevation myocardial infarction.

15 Figure 14 shows a survival analysis of patients following unstable angina/Non-ST elevation myocardial infarction stratifying patients as below or above the median value of plasma N-BNP or of ORP150.

20 Figure 15 shows a survival analysis of patients following unstable angina/Non-ST elevation myocardial infarction, stratifying patients as having both plasma levels of N-BNP and ORP150 below or above the median, and an intermediate group in which either peptide is above their respective medians.

Figure 16 shows the amino acid sequence of human ORP 150.

25 Figure 17 shows the plasma levels of ORP150 and BNP (Brain Natriuretic Peptide-32) in patients undergoing coronary balloon angioplasty.

Example 1

30 *Study Populations*

120 heart failure patients were studied, all with echocardiographically confirmed left ventricular systolic dysfunction (left ventricular (LV) ejection fraction < 45%). A

further 373 patients with myocardial infarction were also recruited. Acute myocardial infarction was defined as presentation with at least two of three standard criteria, i.e. appropriate symptoms, acute ECG changes of infarction (ST elevation, new LBBB), and a rise in creatine kinase (CK) to at least twice the upper limit of normal, i.e. >400 IU/L. 177 of the myocardial infarction patients were also investigated with echocardiography, with systolic function graded as normal, mild, moderate or severe impairment. Age and gender matched normal controls with LV ejection fraction >50%, were recruited from the local community by advertisement. All subjects gave informed consent to participate in the study, which was approved by the local Ethics Committee.

End Points in myocardial infarction patients

End-points were defined as all-cause mortality and cardiovascular morbidity (rehospitalisation with heart failure) following discharge from the index hospitalisation. Multivariate analysis for all endpoints other than death was performed after the censorship of those patients dying during follow up.

Blood Sampling and plasma extraction

In normal subjects and heart failure patients, 20mls of peripheral venous blood was drawn into pre-chilled Na-EDTA (1.5mg/ml blood) tubes containing 500 IU/ml aprotinin after a period of 15 min bed rest. In myocardial infarction patients, a single blood sample was taken between 72-96 hours after symptom onset. After centrifugation at 3000 rpm at 4°C for 15 min, plasma was separated and stored at -70 °C until assay. Prior to assay, plasma was extracted on C₁₈ Sep-Pak (Waters) columns and dried on a centrifugal evaporator. Some urine specimens were also collected from patients with heart failure. These were also extracted on C₁₈ Sep-Pak (Waters) columns as above.

Assay of ORP150

A peptide corresponding to the N-terminal domain (amino acids 33-45) of the human ORP150 sequence (LAVMSVDLGSESM) (Ikeda, *et al*, *Biochem Biophys Res Commun* 1997; **230**: 94-9) was synthesized in the MRC Toxicology Unit, University

of Leicester. Amino acids 1-32 may represent a signal sequence for the protein and may not be present in the mature ORP150 protein. A rabbit was injected monthly with this peptide conjugated to keyhole limpet hemocyanin using maleimide coupling to a cysteine added to the C-terminal of the sequence. IgG from the sera was purified on protein A sepharose columns. The above peptide was also biotinylated using biotin-maleimide in buffer containing (in mmol/l) NaH₂PO₄ 100, EDTA 5, pH 7.0 for 2 hours. After quenching with excess cysteine, the tracer was purified on HPLC using an acetonitrile gradient. Alternatively, the above peptide could be synthesized with incorporation of a biotinylated amino acid at the C- or N-terminus and used as a tracer. Plasma extracts and standards were reconstituted with ILMA (immunoluminometric assay) buffer consisting of (in mmol/l) NaH₂PO₄ 1.5, Na₂HPO₄ 8, NaCl 140, EDTA 1 and (in g/l) bovine serum albumin 1, azide 0.1. ELISA plates were coated with 100 ng of anti-rabbit IgG (Sigma Chemical Co., Poole, UK) in 100 µl of 0.1 mol/l sodium bicarbonate buffer, pH 9.6. Wells were 15 then blocked with 0.5% bovine serum albumin in bicarbonate buffer. A competitive immunoluminometric assay was set up by preincubating 200 ng of the IgG with standards or samples within the wells. After overnight incubation, 50 µl of the diluted biotinylated ORP peptide tracer (2 µl /ml of the stock solution or a total amount of 100-500 fmol) was added to the wells. Following another 24 h of incubation at 4°C, 20 wells were washed 3 times with a wash buffer (NaH₂PO₄ 1.5 mmol/l, Na₂HPO₄ 8 mmol/l, NaCl 340 mmol/l, Tween 0.5 g/l, sodium azide 0.1 g/l). Streptavidin labeled with methyl-acridinium ester (MAE) was synthesized as described (Ng *et al*, *Clinical Science* 2002; **102**: 411-416). Wells were incubated for 2 h with 100 µl of ILMA containing streptavidin-MAE (5 million relative light units per well). Following 25 further washes, chemiluminescence was detected by sequential injections of 100 µL of 0.1 M nitric acid (with H₂O₂) and then 100 µL of NaOH (with cetyl ammonium-bromide) in a Dynatech MLX Luminometer. The lower limit of detection (defined as 3 times standard deviation at zero peptide concentration) was 9.8 fmol per tube or 98 fmol/ml of plasma extracted. Within assay coefficients of variation were 3.1, 4.3 and 30 5.9% for 2, 30, 500 fmol/tube respectively. There was no cross-reactivity with

peptides previously demonstrated to be elevated in heart failure such as ANP, BNP, N terminal proBNP or CNP.

Assay N-BNP

5 The assay for N-terminal proBNP was based on the non-competitive N-terminal proBNP assay described by Karl, *et al*, *Scand J Clin Lab Invest Suppl* 1999; **230**:177-181. Rabbit polyclonal antibodies were raised to the N-terminal (amino acids 1-12) and C-terminal (amino acids 65-76) of the human N-terminal proBNP. IgG from the sera was purified on protein A sepharose columns. The C-terminal directed antibody
10 (0.5 µg in 100 µL for each ELISA plate well) served as the capture antibody. The N-terminal antibody was affinity purified and biotinylated. Aliquots (20 µL) of samples or N-BNP standards were incubated in the C-terminal antibody coated wells with the biotinylated antibody for 24 hours at 4°C. Following washes, streptavidin labeled
15 with methyl-acridinium ester (streptavidin-MAE, 5 x 10⁶ relative light units /ml) (Ng *et al*, *Clinical Science* 2002; **102**: 411-416) was added to each well. Plates were read on a Dynatech MLX Luminometer as previously described (Ng *et al*, *Clinical Science* 2002; **102**: 411-416). The lower limit of detection was 5.7 fmol/ml of unextracted plasma. Within and between assay coefficients of variation were acceptable at 2.3% and 4.8% respectively. There was no cross-reactivity with ANP, BNP or CNP.

20

Size exclusion chromatography and gel electrophoresis of plasma extracts

Plasma extracts were fractionated by isocratic size exclusion chromatography on a 300 x 7.8mm Bio-Sep SEC S2000 column (Phenomenex, Macclesfield, Cheshire, UK) using 50 mmol/l NaH₂PO₄ (pH 6.8) at a flow rate of 1 ml/min as the mobile
25 phase. Standards used to establish molecular weights included IgG (150kD), BSA (68kD), ovalbumin (44kD), soybean trypsin inhibitor (20kD), aprotinin (6.5kD) and tryptophan (204D) (from Sigma Chemical Co, Poole, UK.). Fractions collected every 20 sec were dried on a centrifugal evaporator before assaying for ORP150 as above.

30

Statistical analysis

Statistical analysis was performed using SPSS Version 11.0 (SPSS Inc, Chicago, MI). Data are presented as mean ± SEM or median (range) for data with non-Gaussian

distribution, which were log transformed prior to analysis. For continuous variables, one-way analysis of variance (ANOVA) was used. The interaction of multiple independent variables was sought using the univariate General Linear Model procedure with least significant difference P values reported. Pearson correlation analysis was performed and box plots were constructed consisting of medians, boxes representing interquartile ranges and the whiskers representing the 2.5th to the 97.5th centile. P values below 0.05 were considered significant. Kaplan Meier survival analysis was used to examine the usefulness of peptide levels in risk stratification following MI.

10

Results & Discussion

Performance of the ORP150 assay

A typical standard curve for ORP150 peptide is illustrated in Figure 1, showing a fall in chemiluminescence with increasing concentrations of the peptide. Half displacement of binding of the tracer occurred at about 300 fmol per tube. Dilutions of a heart failure patients' plasma and urine extracts showed parallelism with the standard curve. The lower limit of detection was 9.8 fmol/tube.

20 In addition, isocratic size exclusion chromatography was performed on human plasma extracts (Figure 2). This was resolved into 3 main immunoreactive fractions, one at 150 kD (which is the expected molecular weight of human ORP150 protein), a smaller peak at 6.7 to 7.4 kD and the largest one at 1.8 to 3.3 kD. This suggests that ORP150 extracted from plasma is fragmented and there may be other fragments that
25 could be detected with other epitope specific antibodies.

Conclusions on detection of ORP in humans

Specific immunoassays for ORP have detected the presence of this peptide in plasma and urine. As ORP150 is an endoplasmic reticulum associated protein, this finding is
30 unexpected. Moreover, the immunoreactivity in plasma is derived from several molecular weight forms, suggesting that fragments of ORP150 may be detectable using epitope specific antibodies.

ORP150 in Normal subjects, Heart Failure and Myocardial Infarction

The characteristics of the normal, heart failure (HF) and myocardial infarction (MI) patients are shown in Table 1. Groups were well matched for gender. The normal and HF groups were matched for age, although the MI group was older than the other groups ($P<0.001$). Peptide levels were normalised by log transformation before analysis. Figure 3 shows the N-BNP and the ORP150 levels in the normal, HF and MI patient groups. Using ANOVA, differences in Log N-BNP ($P<0.0005$) and Log ORP150 ($P<0.0005$) was evident between the 3 groups. For N-BNP, both the HF and MI patients' levels were higher than normal ($P<0.0005$ using Tukey's test for multiple comparisons), but levels in HF and MI groups were comparable (P not significant). For ORP150, both the HF and MI patients' levels were higher than normal ($P<0.0005$ using Tukey's test for multiple comparisons). Levels in the HF group were also significantly higher than those in the MI group ($P<0.0005$).

15

Table 1. Patient characteristics in the study. Means [ranges] are reported.

| | Normal Controls | Heart failure patients | Myocardial Infarction patients |
|----------------------------------------|-------------------------|---------------------------|-----------------------------------|
| Number | 180 (59 (32%)female) | 120 (35 (29%) female) | 373 (95 (26%) female) |
| Age (years) | 61.2 [26-81] | 61.4 [20-87] | 65.1 [32-95] |
| Drug therapy | | | |
| Diuretics | - | 98 | 176 |
| β blockers | - | 47 | 283 |
| ACE inhibitors | - | 99 | 220 |
| Aetiology of Cardiomyopathy | | | |
| Ischaemic | - | 80 | 373 |
| Dilated | - | 29 | - |
| Hypertensive | - | 7 | - |
| Valvular | - | 4 | - |

ORP150 in heart failure

20

Within the normal group, there were age dependent changes in N-BNP (correlation coefficient $r = 0.438$, $P<0.0005$). However, ORP150 was not significantly correlated

with age. Combining the normal and HF groups, N-BNP was again correlated with age ($r = 0.306$, $P < 0.0005$) whereas the correlation of ORP150 with age was modest ($r = 0.138$, $P < 0.02$).

5 Figure 4 shows the N-BNP and ORP150 levels in normals and HF, for both gender. Levels of the peptides are elevated in both males and females with HF ($P < 0.0005$ for both, using univariate general linear model (GLM) procedure). The elevation of both peptides in HF is dependent on the severity of HF as judged by the NYHA class. Figure 5 shows that both peptides rise with increasing NYHA class in both gender.

10 For N-BNP, values in normal subjects were different from NYHA class I, II, III and IV ($P < 0.0005$ for all using Tukey's test). For ORP150, values in normal subjects were different from NYHA class I, II, III and IV ($P < 0.002$, 0.0005 , 0.0005 , 0.0005 respectively using Tukey's test).

15 Using the univariate GLM procedure, and entering age as a covariate and gender and NYHA class as factors, analysis of the log normalised N-terminal proBNP levels in the heart failure patients yielded an r^2 of 0.675 for the model ($P < 0.0005$) with age, gender and NYHA class as significant predictive variables ($P < 0.0005$ for all). There was a significant interaction between gender and NYHA class, suggesting that the rise 20 in N-BNP with increasing NYHA class may differ between males and females ($P < 0.007$). A similar analysis performed on the log normalised ORP150 data yielded an r^2 of 0.512 for the model ($P < 0.0005$) with NYHA class only as a significant predictive variable ($P < 0.0005$). Age and gender were not significant predictive variables, although there was a significant interaction between gender and NYHA 25 class ($P < 0.001$) again suggesting that the rise in ORP150 with increasing NYHA class differs between males and females. Although the majority of HF patients have ischaemic heart disease as the aetiology, detection of HF using these peptides is achieved irrespective of aetiology.

30 Stepwise logistic regression analysis was employed to predict absence or presence of HF, with log N-BNP and log ORP150 as predictive variables. Age and gender were not used since the normal and HF groups were age and gender matched. Both N-BNP

(Odds ratio for 50% rise in peptide level 1.56, $P<0.0005$) and ORP150 (Odds ratio for 50% rise in peptide level 2.46, $P<0.0005$) were independent predictors of presence of HF, accounting for a total r^2 (Cox and Snell) of 0.55 and a Nagelkerke r^2 of 0.74 irrespective of whether forward or backward stepwise procedures was used. The 5 prognostic index (probability of membership of HF group) derived from the above model was used to construct a receiver operating characteristic (ROC) curve (Figure 6). The ROC area for the model was 0.95, greater than that of N-BNP (0.91) or ORP (0.84) alone, for the identification of HF.

10 *Conclusions on ORP150 in Heart Failure*

These findings suggest that although both N-BNP and ORP150 are elevated in HF (and with increasing severity of HF), N-BNP is more affected by age and gender of the subjects (with higher levels with rising age and in females). ORP150 by contrast does not have an age dependent component and is modestly affected by gender. Both 15 peptides are effective in identification of HF, but the combination of the two may have added potential in diagnosis of HF.

ORP150 in myocardial infarction

The patient characteristics of the myocardial infarction (MI) group are shown in Table 20 1, and although gender matched were slightly older than the normal group ($P<0.0005$). Both N-BNP and ORP150 were elevated in the plasma obtained 2-3 days after myocardial infarction ($P<0.0005$ for both, figure 3). Levels of N-BNP were correlated with the peak creatine kinase level ($r = 0.24$, $P<0.0005$) suggesting a relation to the size of the infarction. However, ORP150 levels were not significantly 25 correlated to the peak creatine kinase level ($r = 0.05$, P not significant).

N-BNP was correlated to both age ($r = 0.39$, $P<0.0005$) and creatinine ($r = 0.38$, $P<0.0005$), the partial correlation coefficients remaining significant after allowing for the effects of gender and infarction (with age ($r = 0.39$, $P<0.0005$) and with creatinine 30 ($r = 0.36$, $P<0.0005$)). In contrast, ORP150 was not significantly correlated with age, but weakly with creatinine ($r = 0.20$, $P<0.0005$), the partial correlation coefficient

falling further after allowing for the effects of gender and infarction (with creatinine (r = 0.12, $P<0.007$).

5 The determinants of log normalised ORP150 were sought using stepwise linear regression analysis with age and creatinine as covariates, and presence of MI and gender as factors. Only presence of MI ($P<0.0005$) and creatinine ($P<0.004$) were identified as significant independent predictors of ORP150 levels, accounting for 14% of total variance ($P<0.0005$). A similar analysis with N-BNP levels identified age, gender, creatinine and presence of MI as significant independent predictors ($P<0.0005$ for all). Thus this finding confirms that in the HF group, i.e. ORP150 levels are less 10 susceptible to influence by age and gender than N-BNP levels.

15 We used logistic regression analysis to predict presence or absence of MI as the dependent variable, using age, gender, N-BNP and ORP150 as independent variables. All 4 were identified as independent predictive variables for presence or absence of MI using both forward and backward stepwise regression analysis, the model accounting for an r^2 of 0.56 (Cox and Snell) or 0.79 (Nagelkerke). The odds ratios were as follows:- for N-BNP (for a 50% rise in the peptide level 1.94, $P<0.0005$); for ORP150 (for a 50% rise in the peptide level 1.61, $P<0.0005$).
20

25 The plasma level of N-BNP was related to the Killip class of the patient (figure 7, $P<0.0005$). In contrast, levels of ORP150 were elevated in all MI patients irrespective of Killip class (figure 7). Of the 177 patients who had echocardiography scans, we found that the N-BNP levels was related to degree of LV dysfunction (figure 8, $P<0.0005$). In contrast, ORP150 levels were elevated in all MI patients irrespective of degree of LV dysfunction and even patients who had apparently "normal" LV function had elevated ORP150 levels (figure 8).

Outcomes after MI

30 All cause mortality and readmission rates with heart failure following MI were examined, to investigate the usefulness of ORP150 in prediction of these outcomes. Mean length of follow-up after discharge was 426 days with a range of 5-764 days.

Out of the 367 cases, there were 39 deaths during the follow up period. There were also 22 readmissions with heart failure.

5 Patients who died had significantly higher N-BNP and ORP150 levels ($P<0.0005$ and $P<0.001$ respectively, figure 9). In addition, both peptides were elevated in those patients who were later readmitted with heart failure ($P<0.0005$ for N-BNP, $P<0.025$ for ORP150, figure 10).

10 Logistic regression analysis was used to investigate the predictors of death as an outcome with age, creatinine, past medical history of infarction, Killip class, and log N-BNP or Log ORP150. Significant independent predictors for death included N-BNP (odds ratio for 10 fold rise in peptide level 3.95, $P<0.002$) and ORP150 (odds ratio for 10 fold rise in peptide level 4.58, $P<0.05$), accounting for a Nagelkerke r^2 of 0.32. Backward and forward regression analysis confirmed these two independent 15 predictor variables, but with an additional contribution from creatinine (odds ratio for 10 fold rise 19.56, $P<0.05$). These findings suggest that ORP150 is a predictor of mortality after MI independent of N-BNP levels.

20 Kaplan Meier survival analysis was performed to confirm these findings. When subjects were divided in infra and supra median groups, survival differed significantly between these 2 groups (figure 11), whether the peptide used was N-BNP ($P<0.0005$ by log rank test for trend) or ORP150 ($P<0.002$ by log rank test for trend). Of note is that, even in the infra median groups defined by N-BNP or ORP150 alone, there is a definite mortality rate (albeit slower than the supra median groups). We utilised the 25 ranks in both the N-BNP and ORP150 ranked groups to yield a novel prognostic index, with patients divided up into 3 groups (both peptides below the medians, either peptide above the medians and both peptides above the medians). Figure 12 shows the survival analysis using this new prognostic index, showing no deaths during the observational period in the group with both peptides below the medians, a high 30 mortality rate in those with both peptides above the medians, and an intermediate mortality rate in those with either peptide above the medians ($P<0.0005$ by log rank test for trend).

Conclusions on ORP in MI

Plasma ORP150 levels are elevated in ischaemic heart disease as manifested by myocardial infarction. In contrast to N-BNP which is also elevated in these patients, 5 ORP150 levels are less dependent on age, degree of LV dysfunction, symptoms and signs (as determined by Killip class) and renal function. Both peptides are good predictors of outcomes such as mortality or readmission with heart failure following the index admission with myocardial infarction. In particular, the combination of both peptides may be particularly useful in risk stratification after myocardial 10 infarction (prediction of mortality).

Overall Conclusions on ORP in vascular disease

The above data demonstrates that ORP150 is secreted into human plasma and can also be found in urine. There may be fragments of ORP150 in bodily fluids. The levels of 15 ORP150 are elevated in both Heart Failure and Ischaemic Heart Disease, and the measurement may be less prone to age and gender interference. As atherosclerosis is the major cause of vascular disease, ORP150 may be of use in the diagnosis or prognosis of other conditions where there is tissue hypoxia, for example, strokes, peripheral vascular disease, aneurysms, or acute coronary syndromes. In Heart 20 Failure, in addition to being a diagnostic aid in itself, it could complement the measurement of N-BNP. In Myocardial Infarction, it may serve as an indicator of prognosis, predicting both death and readmissions with heart failure. Independently or in combination with N-BNP, its measurement after myocardial infarction is an effective aid to risk stratification able to detect extremely low or high risk groups of 25 patients. This may have impact in the planning of therapeutic options for patients.

Example 2

A further 114 patients with unstable angina or non-ST elevation myocardial infarction 30 (subendocardial myocardial infarction, defined as a rise of creatine kinase of under 2 fold upper limit of normal) were studied. All patients had chest pain at rest and were admitted to hospital for treatment. The mean (range) age was 66.8 years (38-93) and

there were 74 men, 40 women. Blood samples were obtained at 3-5 days after admission to hospital, and analysed for troponin-T (Roche Diagnostics), ORP150 protein and N-BNP as detailed in Example 1.

5 Patients were followed up for end-points as described for myocardial infarction patients in Example 1.

During the mean follow up period of 401 days (range 26-764 days), there were 9 deaths. Troponin-T levels were not significantly different in those who died (0.12 10 (0.005-1.14) $\mu\text{g/L}$) compared to those who survived (0.19 (0.005-0.557) $\mu\text{g/L}$).

In contrast, both ORP150 and N-BNP levels were significantly higher in those who died compared to survivors ($P<0.006$ and $P<0.05$ respectively, figure 13).

15 Kaplan Meier survival analysis was performed using both N-BNP and ORP150 levels (below or above median) for case stratification. When subjects were divided in infra and supra median groups, survival differed significantly between these 2 groups (Figure 14), whether the peptide used was N-BNP ($P<0.016$ by log rank test for trend) or ORP150 ($P<0.015$ by log rank test for trend). When both peptides are used to 20 classify patients into 3 groups (both peptides below the medians, either peptide above the medians and both peptides above the medians), the survival analysis suggested that those patients with both peptides below median had no deaths during the observational period, whereas those with both peptides above the median had a high mortality rate ($P<0.002$ by log rank test for trend, figure 15). This novel prognostic 25 index for unstable angina/non-ST elevation myocardial infarction thus enables risk stratification similar to that described in the patients with ST-elevation myocardial infarction (as described above).

Conclusions on ORP in Unstable angina/Non-ST elevation MI

30 Plasma ORP150 and N-BNP levels are elevated in ischaemic heart disease as manifested by unstable angina/Non-ST elevation MI. Both peptides are good predictors of outcomes such as mortality. In particular, the combination of both

peptides may be particularly useful in risk stratification after unstable angina/Non-ST elevation MI (prediction of mortality). Use of such a prognostic index would enable treatment of the patients at highest risk of mortality with revascularisation or pharmacological agents.

5

Example 3

The effect of acute obstruction to the coronary circulation during balloon angioplasty was evaluated in 19 patients with coronary artery disease, who were undergoing this therapeutic procedure for treatment of atherosclerosis. Plasma was collected before the procedure, and at 2 hours, 6 hours and 12 hours after the angioplasty. The level of ORP150 was measured as described above. Additionally, the level of a known cardiac marker of ventricular wall stress which is known to be elevated after other coronary occlusion events such as myocardial infarction, namely B-type or Brain 10 natriuretic peptide (BNP) was measured in C₁₈ column using an Immunoluminometric assay. 15

Figure 17 illustrates the changes in plasma ORP150 levels after angioplasty compared to BNP. Both markers significantly change with time (P<0.001 using the analysis of variance with repeated measures). In addition, the plasma levels of both peptides peak at 2 hours after angioplasty, falling beyond that time back to baseline levels. Peak ORP150 levels at 2 hours were significantly different from basal (P<0.02) and 6 and 12 hour levels (P<0.001 for both). For BNP, peak levels at 2 hours were different from basal (P<0.001) and 6 and 12 hour levels (P<0.005 for both). 20 25

The rapid increase in ORP150 levels after balloon occlusion suggests that it can be used as an indicator of acute occlusion of the coronary circulation, as in myocardial infarction or other acute coronary syndromes (e.g. non ST elevation myocardial infarction or unstable angina).

Claims

1. A method for screening, diagnosis or prognosis of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for
5 determining the stage or severity of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for identifying a mammalian subject at risk of developing tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, or for monitoring the effect of therapy administered to a mammalian subject having tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, said method
10 comprising:

measuring the level of a first marker in a sample of bodily fluid from said mammalian subject, wherein said first marker is oxygen regulated protein (ORP150) or a fragment thereof.

15 2. A method as claimed in claim 1, wherein the bodily fluid is plasma.

3. A method as claimed in claim 1 or claim 2, wherein the level of the first marker is compared with the level of the first marker from one or more mammalian subjects free from tissue hypoxia or a clinical syndrome indicative of tissue hypoxia,
20 or with a previously determined reference range for the first marker in mammalian subjects free from tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia.

4. A method as claimed in claim 1, 2 or 3, wherein the level of the first marker is
25 measured by contacting the sample with an antibody that is immunospecific for the first marker and measuring any binding that has occurred between the antibody and at least one species in the sample.

30 5. A method as claimed in claim 4, wherein the antibody is a monoclonal antibody.

6. A method as claimed in any preceding claim, wherein the clinical syndrome indicative of tissue hypoxia is heart failure or ischaemic heart disease.
7. A method as claimed in claim 6, further comprising measuring the level of a second marker indicative of heart failure or ischaemic heart disease.
5
8. A method as claimed in claim 7, wherein the second marker is a natriuretic peptide.
- 10 9. A method as claimed in claim 8, wherein the natriuretic peptide is brain natriuretic peptide (BNP) or N-terminal pro-brain natriuretic peptide (N-BNP).
- 15 10. A method as claimed in claim 7, 8 or 9, wherein the level of the second marker is compared with the level of the second marker from one or more mammalian subjects free from tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, or with a previously determined reference range for the second marker in mammalian subjects free from tissue hypoxia or a clinical syndrome indicative of tissue hypoxia.
15
- 20 11. A method as claimed in any one of claims 7 to 10, wherein the level of the second marker is measured by contacting the sample with an antibody that is immunospecific for the second marker and measuring any binding that has occurred between the antibody and at least one species in the sample.
- 25 12. A method as claimed in claim 11, wherein the antibody is a monoclonal antibody.
25
- 30 13. A method as claimed in any one of claims 1 to 5, wherein the clinical syndrome indicative of tissue hypoxia is atherosclerosis, ischaemic stroke, aortic aneurysm, peripheral vascular disease, lung disease or tumour growth.
30
14. A kit for carrying out the method of any preceding claim.

15. A kit for screening, diagnosis or prognosis of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for determining the stage or severity of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for identifying a mammalian subject at risk of developing tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, or for monitoring the effect of therapy administered to a mammalian subject having tissue hypoxia or a clinical syndrome indicative of tissue hypoxia, said kit comprising:

5 instructions for taking a sample of bodily fluid from said mammalian subject; and

10 one or more reagents for measuring the level of oxygen regulated protein (ORP150) or fragment thereof in the sample.

16. A kit as claimed in claim 15, wherein the bodily fluid is plasma.

15 17. A kit as claimed in claim 15 or claim 16, wherein the one or more reagents comprise an antibody that is immunospecific for the first marker.

18. A kit as claimed in claim 17, wherein the antibody is a monoclonal antibody.

20 19. A kit as claimed in any one of claims 15 to 18, wherein the clinical syndrome indicative of tissue hypoxia is heart failure or ischaemic heart disease.

25 20. A kit as claimed in claim 19, further comprising one or more reagents for measuring the level of a second marker indicative of heart failure or ischaemic heart disease.

21. A kit as claimed in claim 20, wherein the second marker is a natriuretic peptide.

30 22. A kit as claimed in claim 21, wherein the natriuretic peptide is brain natriuretic peptide (BNP) or N-terminal pro-brain natriuretic peptide (N-BNP).

23. A kit as claimed in claim 20, 21 or 22, wherein the one or more reagents for measuring the second marker comprises an antibody that is immunospecific for the second marker.

5 24. A kit as claimed in claim 23, wherein the antibody is a monoclonal antibody.

25. A kit as claimed in any one of claims 15 to 18, wherein the clinical syndrome indicative of tissue hypoxia is atherosclerosis, ischaemic stroke, aortic aneurysm, peripheral vascular disease, lung disease or tumour growth.

AbstractBodily fluid markers of tissue hypoxia

5 The present invention provides a method and kit for screening, diagnosis or prognosis of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for determining the stage or severity of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia in a mammalian subject, for identifying a mammalian subject at risk of developing tissue hypoxia or a clinical syndrome

10 indicative of tissue hypoxia, or for monitoring the effect of therapy administered to a mammalian subject having tissue hypoxia or a clinical syndrome indicative of tissue hypoxia. The level of oxygen regulated protein (ORP150) or a fragment thereof in a sample of bodily fluid from said mammalian subject is measured to provide information as regards the tissue hypoxia or clinical syndrome indicative of tissue

15 hypoxia.

Figure 1

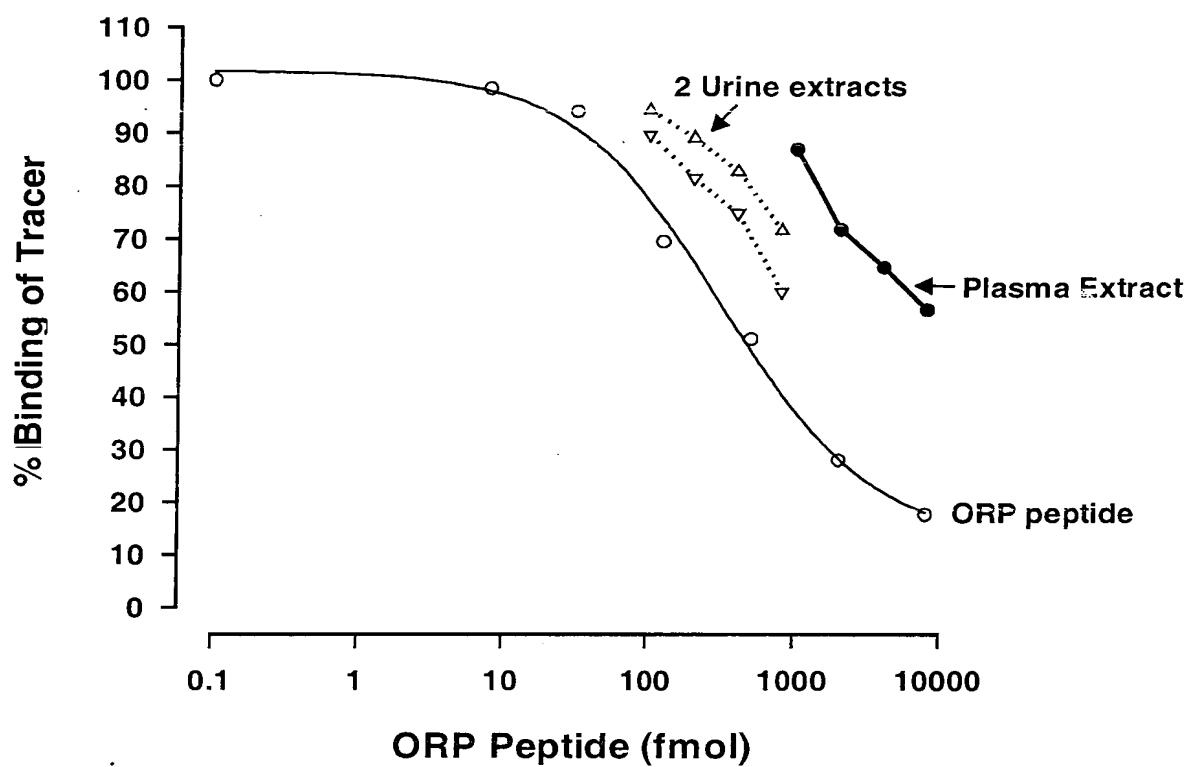


Figure 2

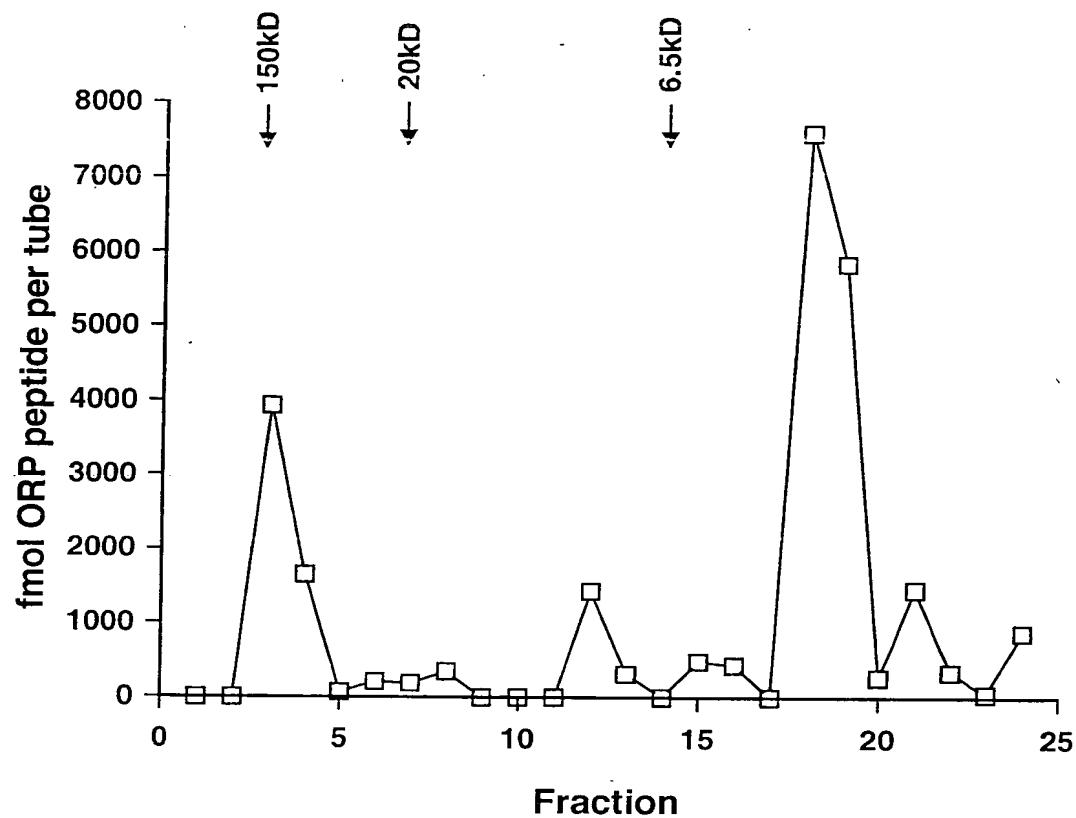


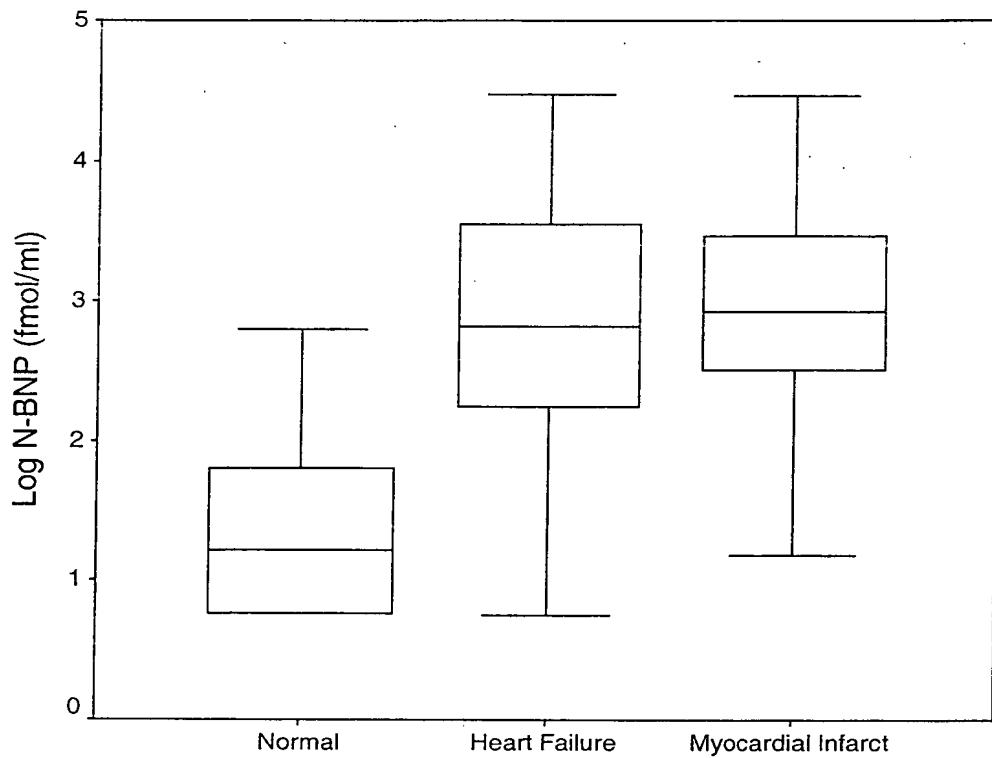
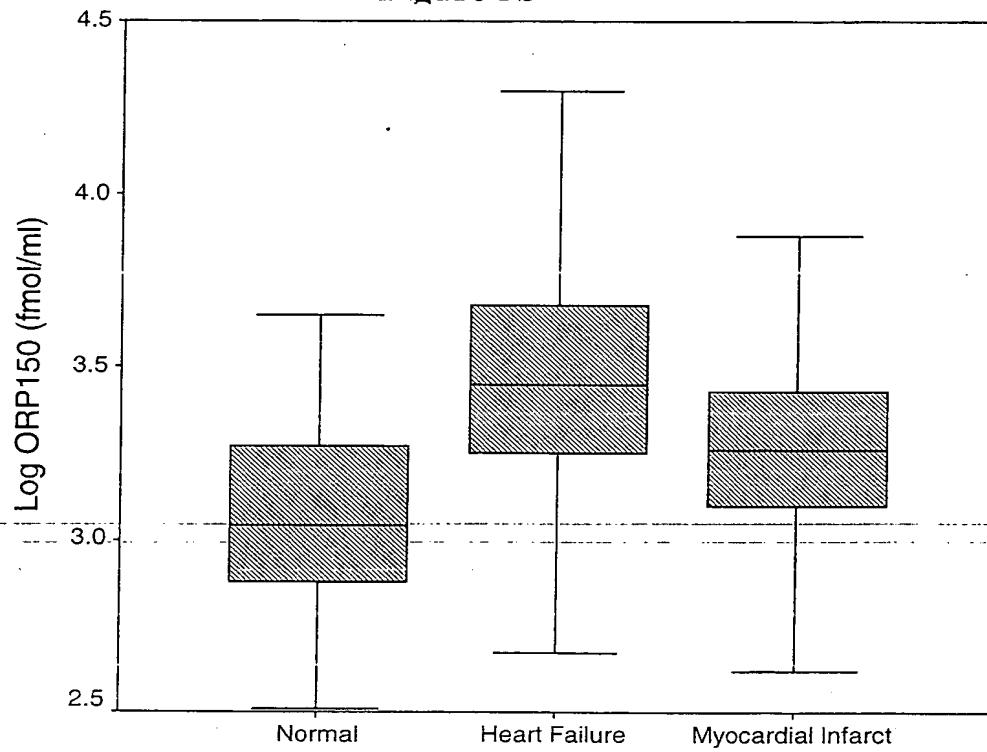
Figure 3a**Figure 3b**

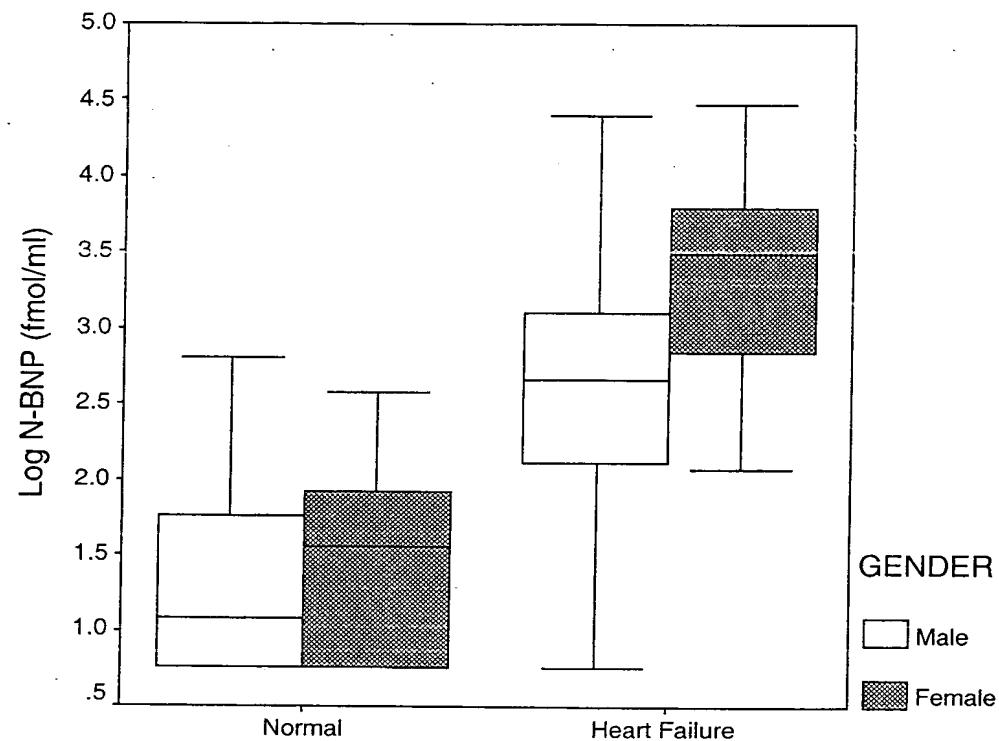
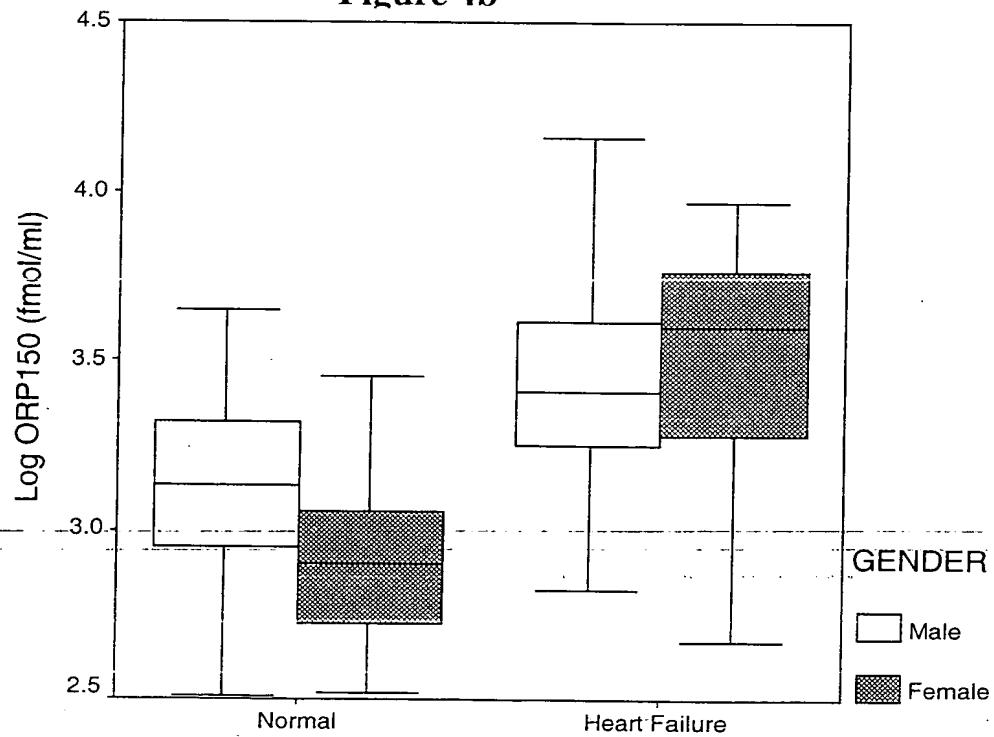
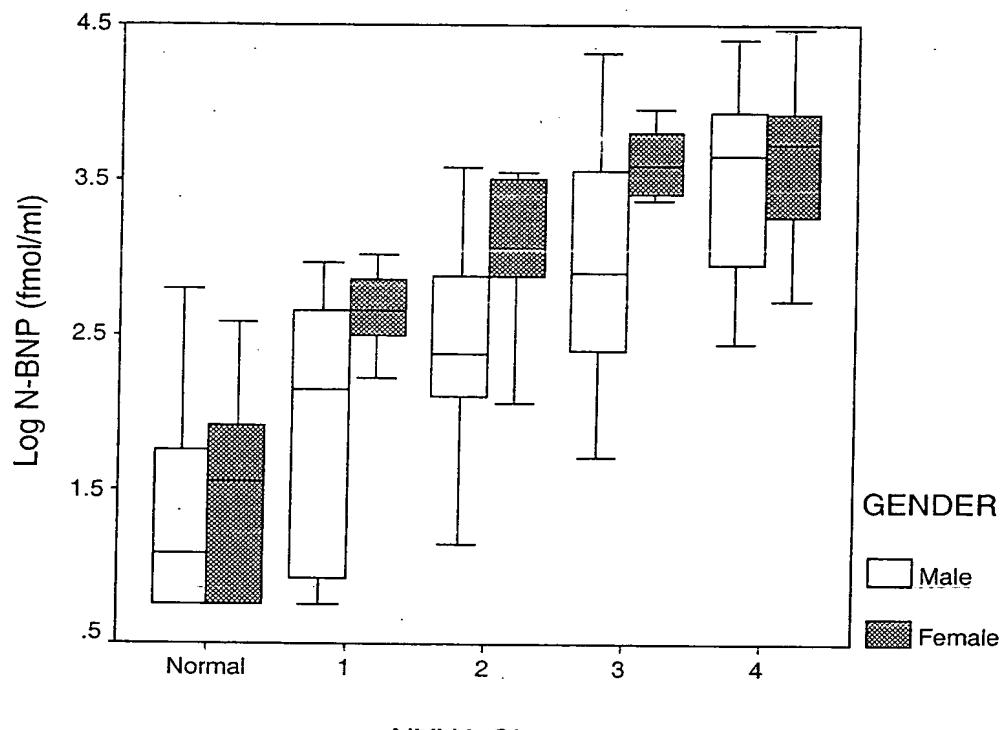
Figure 4a**Figure 4b**

Figure 5a

NYHA Class

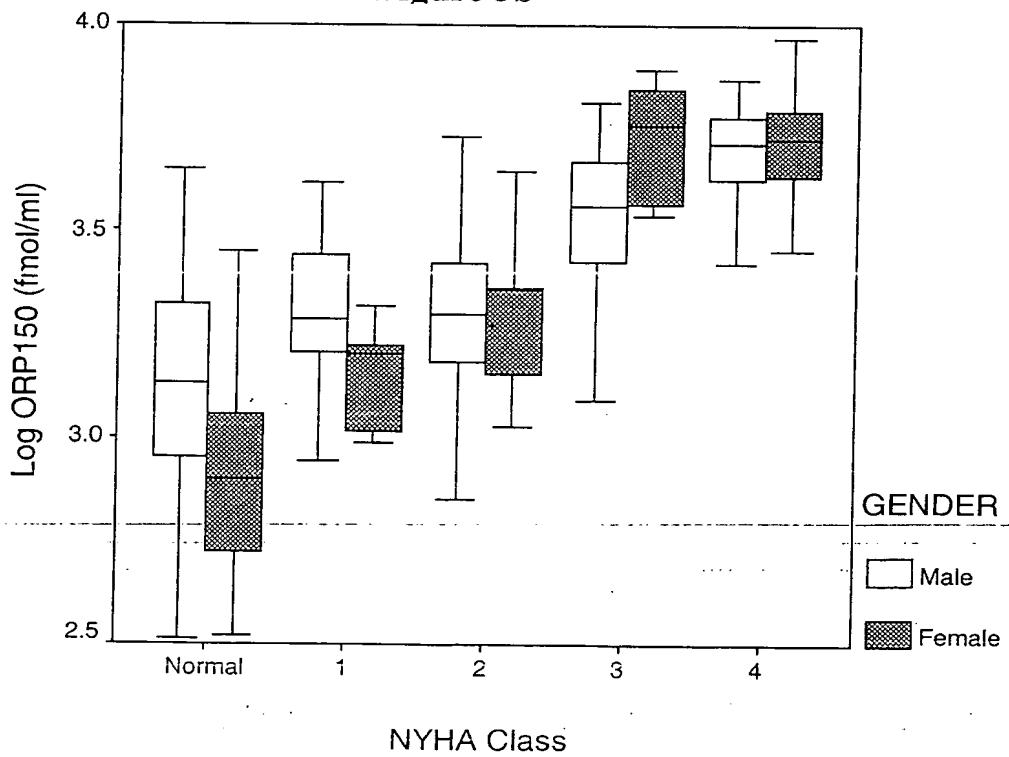
Figure 5b

Figure 6

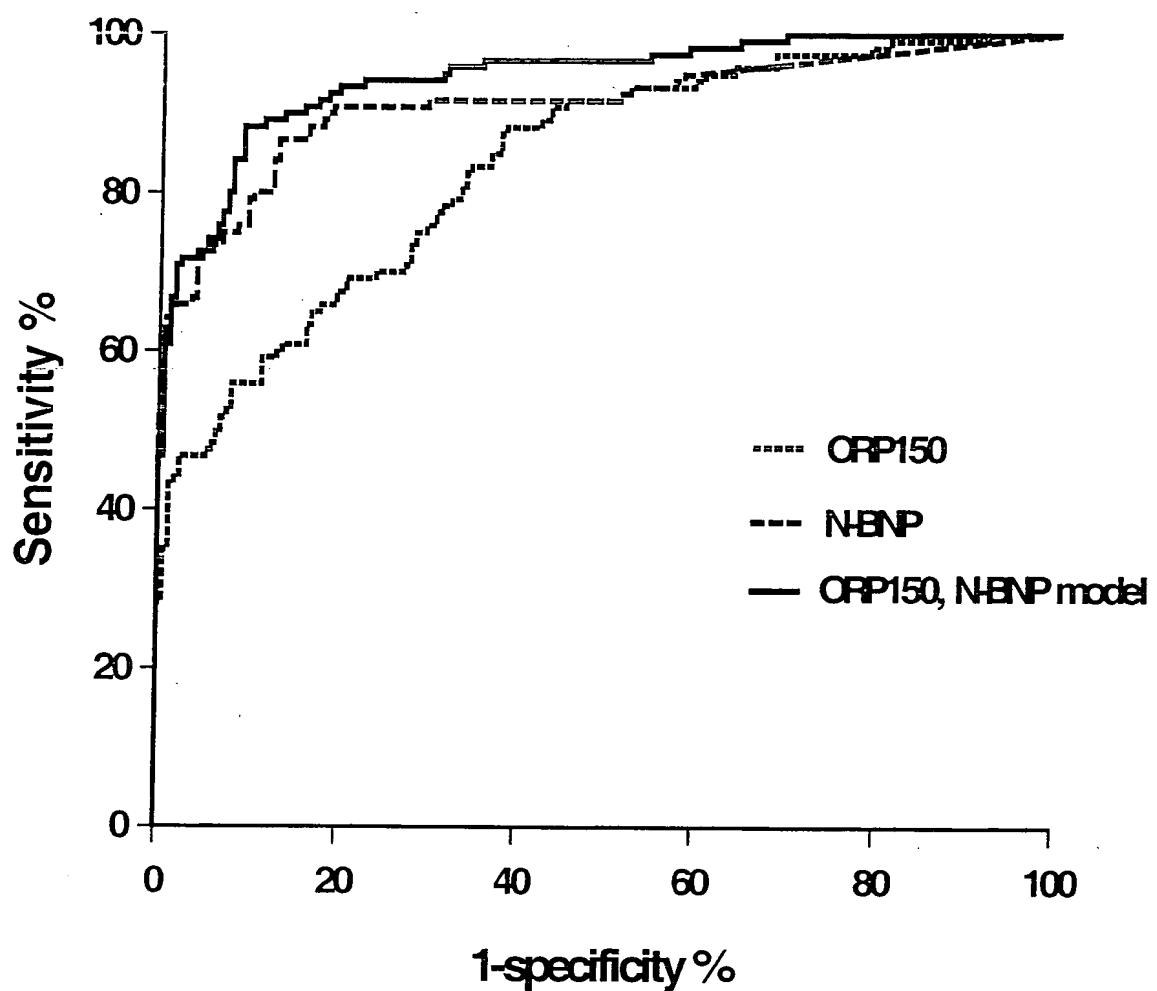


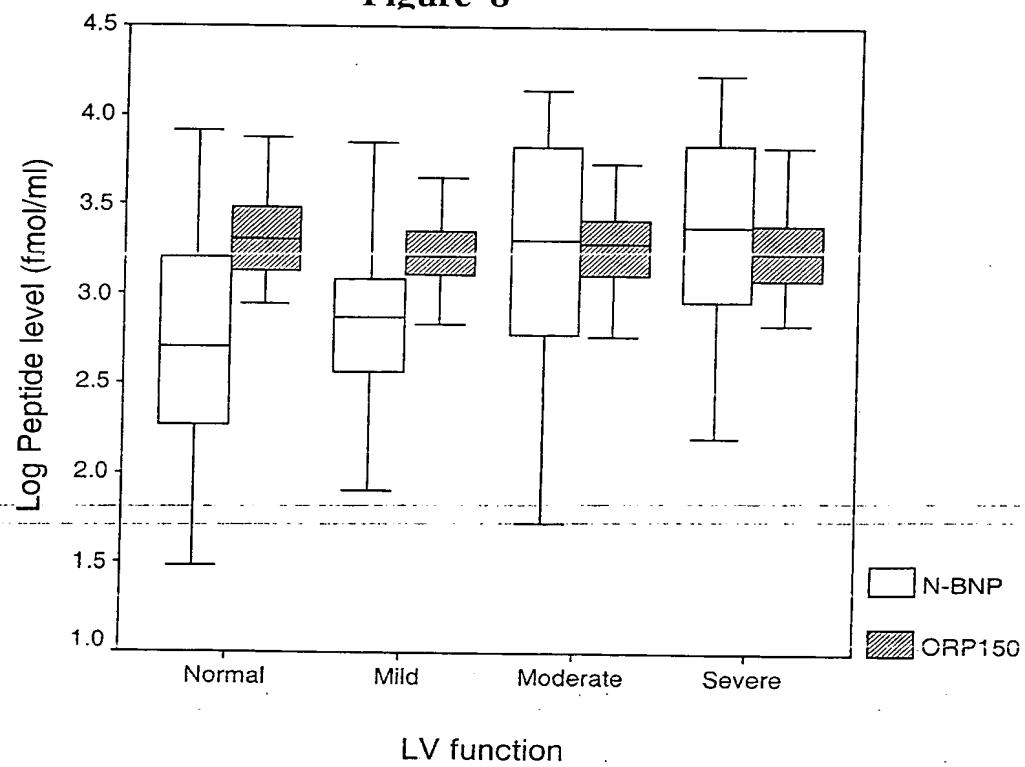
Figure 7**Figure 8**

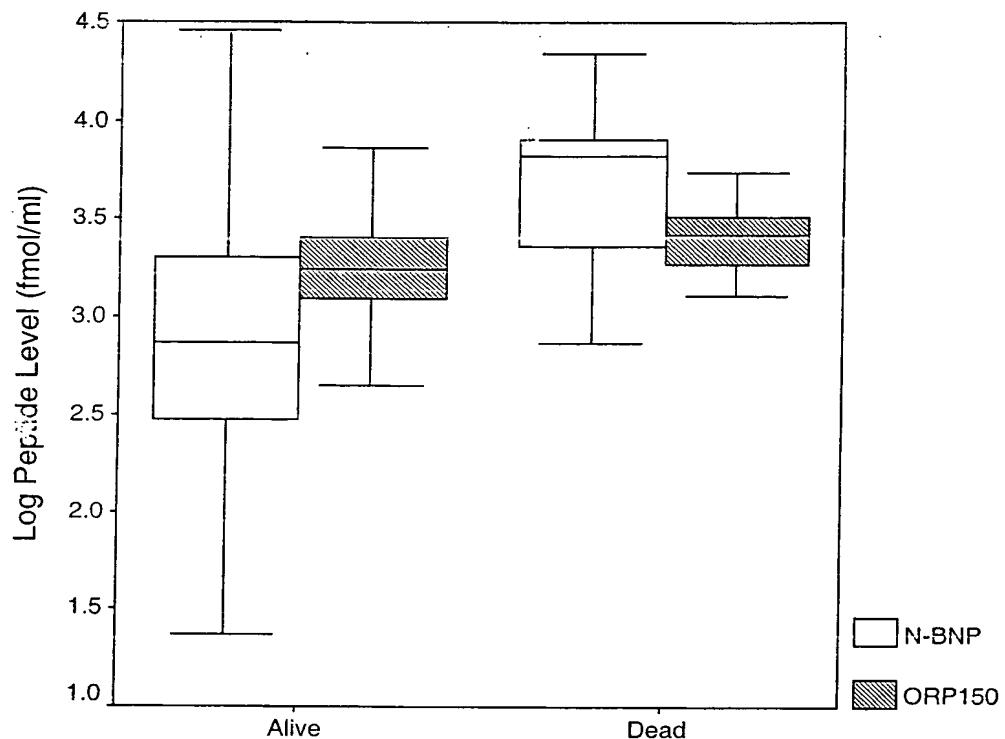
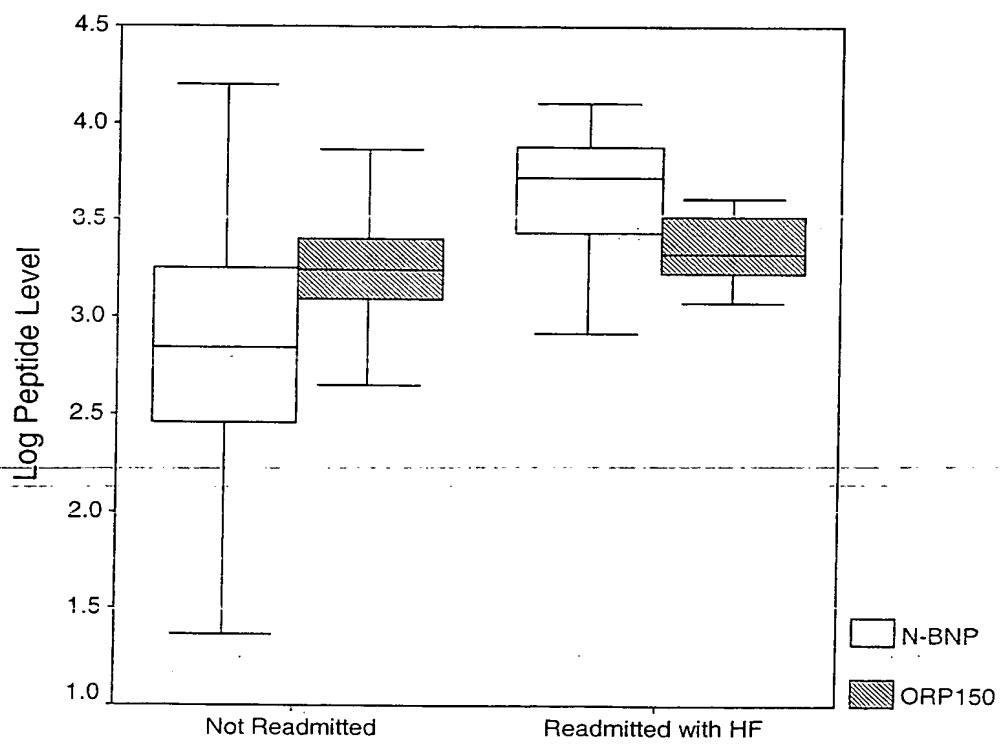
Figure 9**Figure 10**

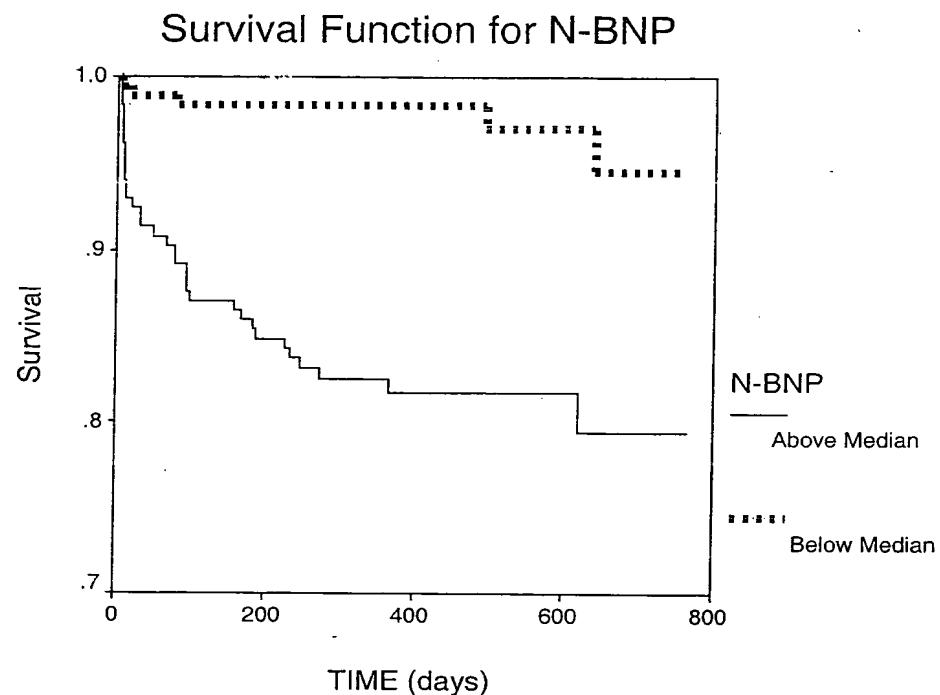
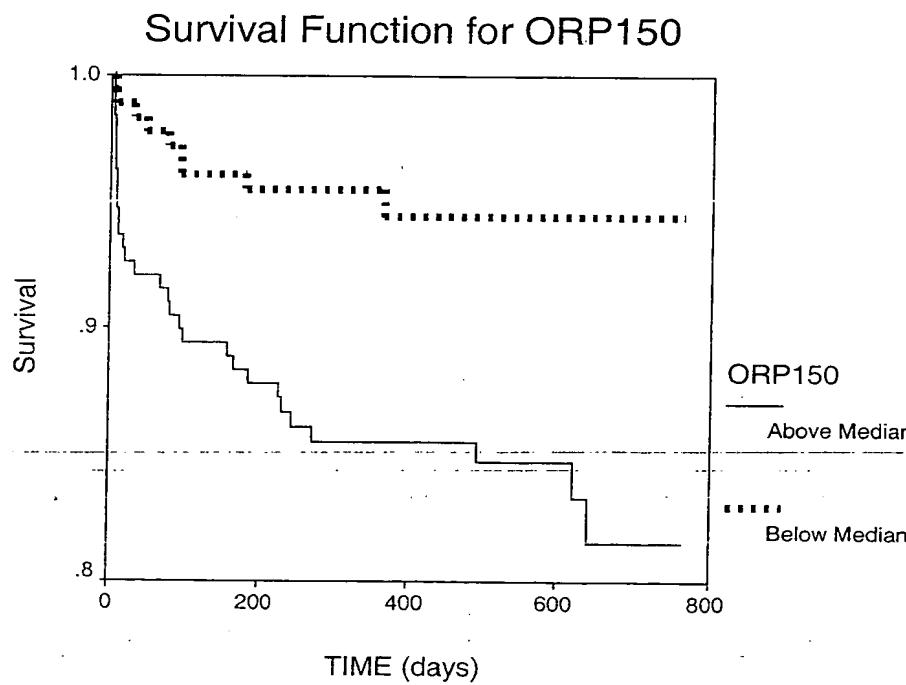
Figure 11a**Figure 11b**

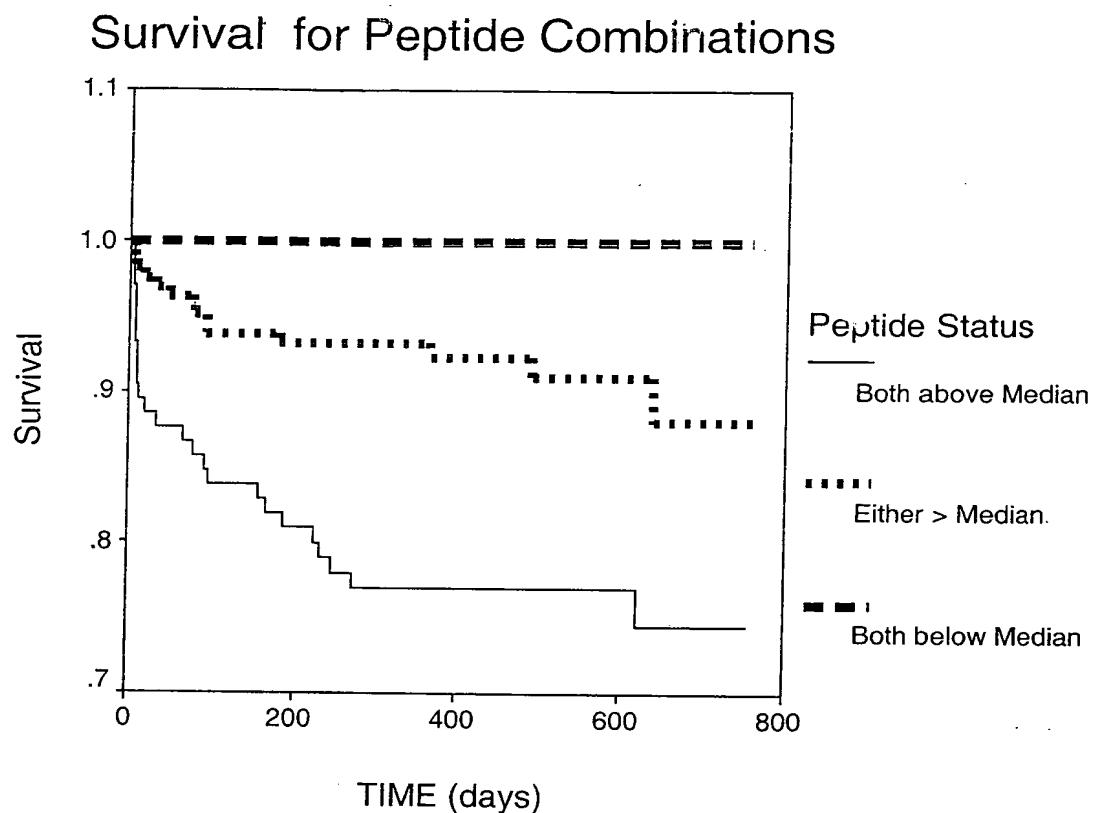
Figure 12

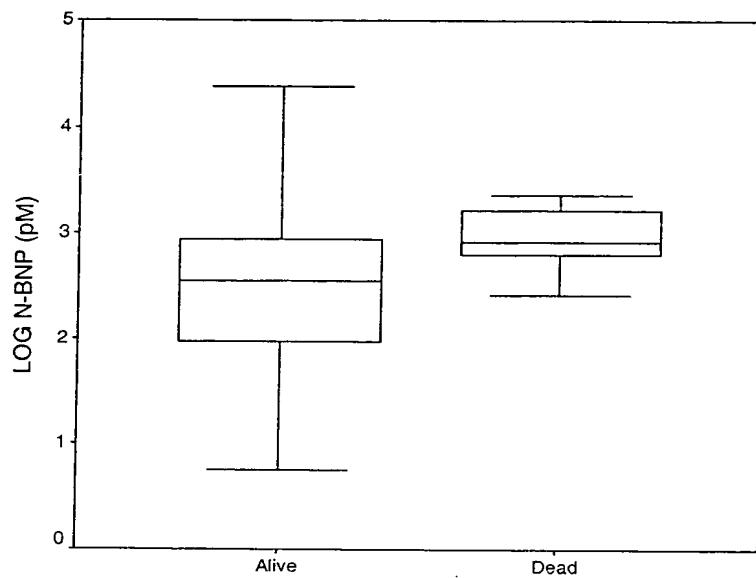
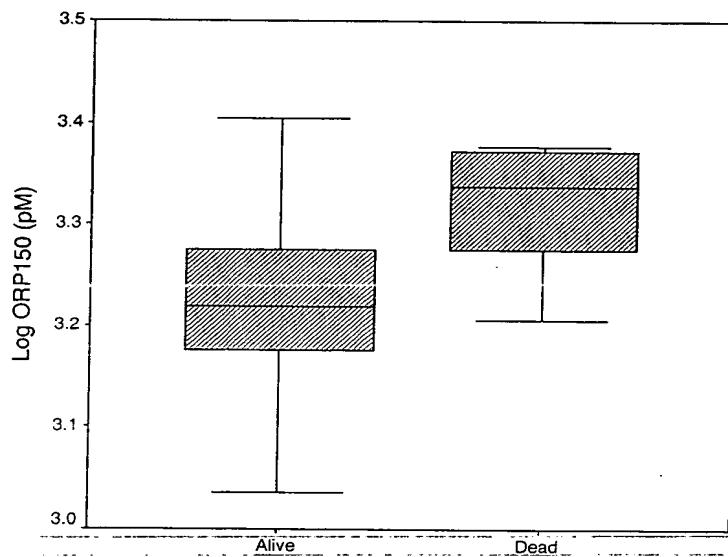
Figure 13a**Figure 13b**

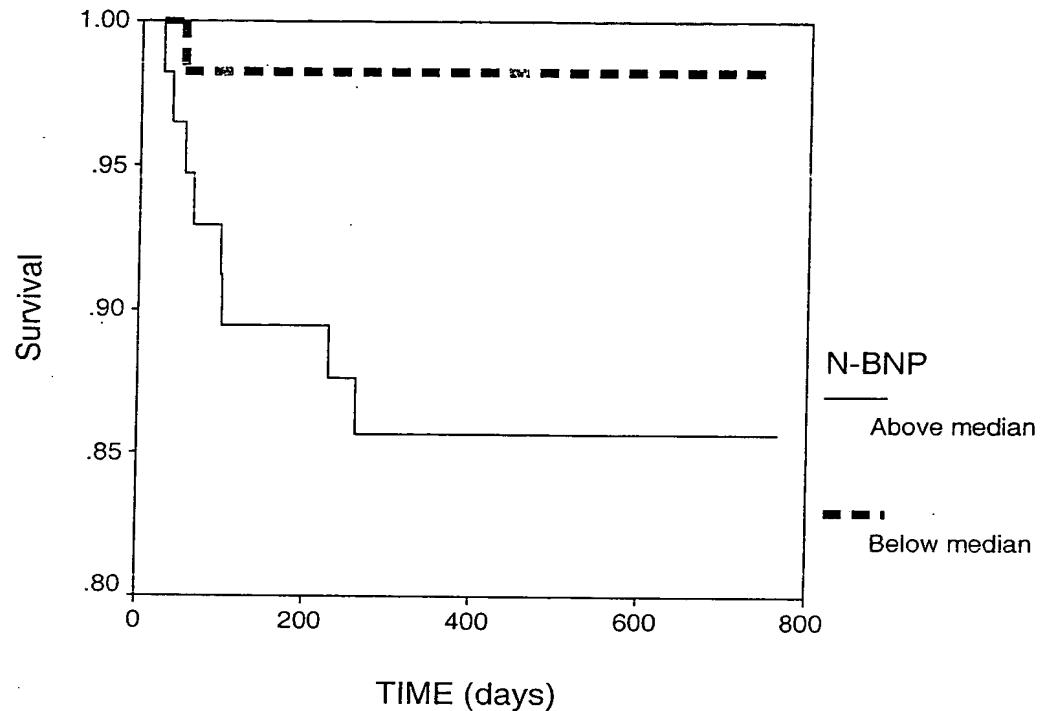
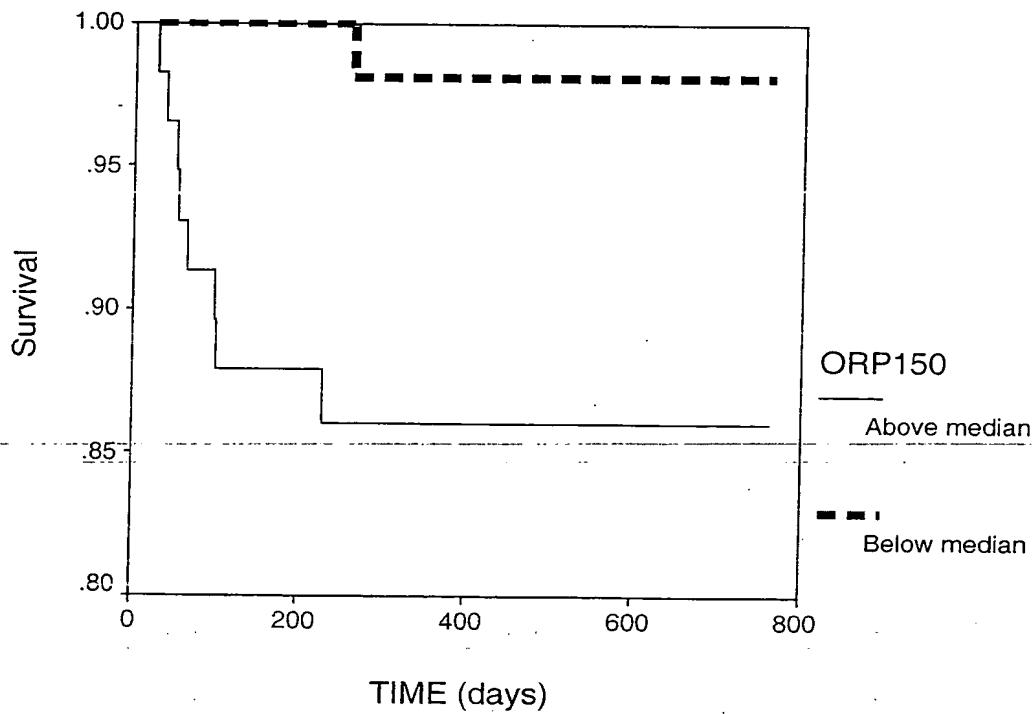
Figure 14a**Survival Function for N-BNP****Figure 14b****Survival Function for ORP150**

Figure 15
Survival Functions N-BNP and ORP150

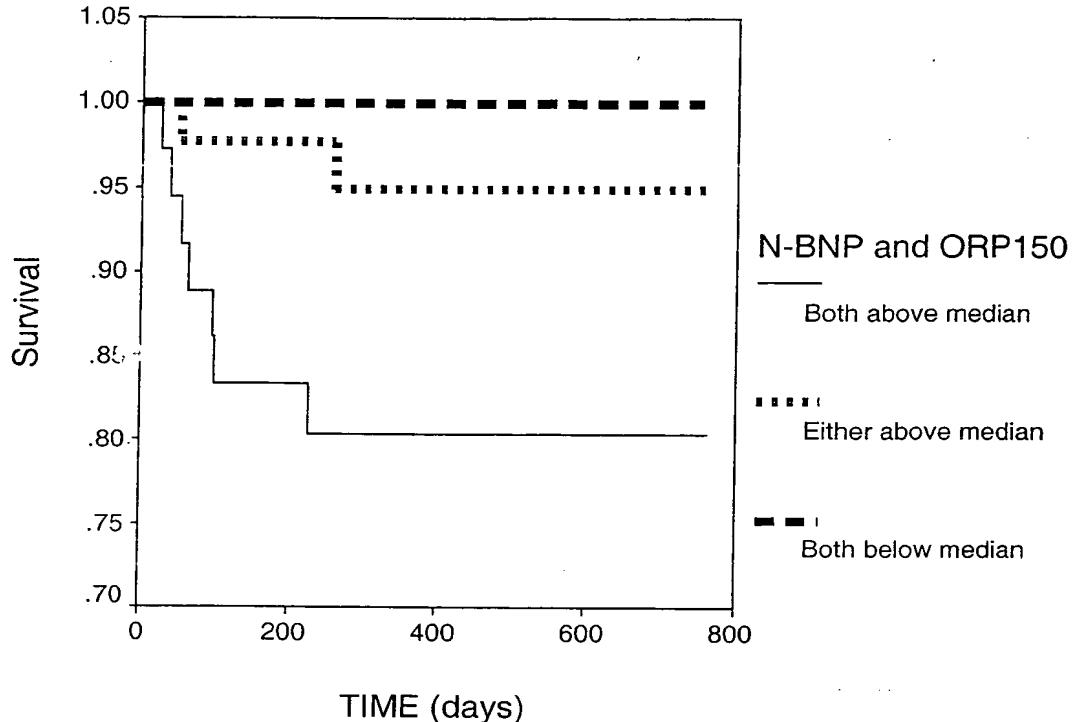


Figure 16

1 madkvrrqrp rrrvcwalva vlladllals dtlavmsvdl gsesmkvaiv kpgvpmeivl
 61 nkesrrktpv ivtlkenerf fgdsasmai knpkat1ryf qh11gkqadn phvalyqarf
 121 peheltfdpq rqtvhfhqiss qlqfspeevl gmvlneysrsl aedfaeqpik davitpvff
 181 nqaerravlg aarmaglkvl qlindntata lsygvfrkd inttaqnimf ydmgsgstvc
 241 tivtyqmvkt keagmqpqlq irgvgfdrtl gglemelrlr erlaglfneq rkgqrakdvr
 301 enpramakll reanrlktvl sanadhmaqi eglmddvdfk akvtrvfeefee lcadlfervp
 361 gpvqqalqsa emsldeieqv ilvggatrvp rvqevllkav gkeelgknin adeaaamgav
 421 yqaaalskaf kvkpfvvrdv vvypilveft reveeeepgih slkhnkrvlf srmgpypqrk
 481 vitfnryshd fnfhinygdl gflgpedlrv fgsqnltvk lkgvgdsfkk ypdyeskgik
 541 ahfnldesgv lsldrvvesvf- etlvedsaee estl-tklignt- iss1f9ggtt- pdakengtdt-
 601 vqeeeeespa gskdepgeqv elkeeeaeapv edgsqpppve pkgdatpege katekengdk
 661 seaqkpseka eagpegvapa pegekkqkpa rkrrmveeig velvvldlpd lpedklaqsv
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 781 wledegvgat tvmlkeklae lrklcqglff rveerkkwpe rlsaldnlln hssmf1kgar
 841 lipemdqift evemttlekv inetwawkna tlaeqaklpa tekpvllskd ieakmmaldr
 901 evqy1lnkak ftkprprpkd kngtraepll nasasdqgek vippagqted aepisepekv
 961 etgsep1gdte plelggpgae peqkeqstgq krplkndel

Figure 17

